IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Leonard H. Rome, Valerie A. Kickhoefer, Sujna Raval-Fernandes, Phoebe L. Stewart

Confirmation No.: 1636

Application No.: 10/547,530

Group Art Unit: 1636

Filing Date: August 31, 2005

Examiner: David Guzo

For: VAULT AND VAULT-LIKE CARRIER MOLECULES

DECLARATION OF LEONARD H. ROME, PH.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Leonard H. Rome, Ph.D., under penalty of perjury, declare as follows:

1. I am an inventor of the subject matter claimed in U.S. Patent Application No. 10/547,530 ("'530 Application"), and I am familiar with its content and teachings.

2. I hold a B.S. in Chemistry and M.S. and Ph.D. degrees in Biological Chemistry from the University of Michigan, and I am currently a professor in the Department of Biological Chemistry and the Senior Associate Dean for Research at the School of Medicine at the University of California Los Angeles (UCLA). I have studied vaults extensively since 1985 when the particle was discovered in my laboratory at UCLA, and have authored numerous vault-related research articles and publications. A copy of my curriculum vitae is attached as Exhibit A.

3. Experiments have been conducted under my direct control in which vault-like particles (VLPs) were made and used according to the claims of the '530 Application. The experiments were initiated in the months immediately following the priority date of the '530 Application (March 10, 2003), and were carried out following the teachings of the '530

Application, using known methods and the routine optimization of experimental conditions. The results of the experiments were published in an article in the journal *Proceedings of the National Academy of Sciences*, which is attached as Exhibit B.

4. The experiments included making and administering VLPs comprising: i) a luciferase polypeptide linked to the INT domain from the C-terminus of VPARP; ii) a GFP variant polypeptide (GL) linked to the same INT domain from the C-terminus of VPARP; and iii) the GL polypeptide linked to MVP. The VLPs were made using a recombinant baculovirus expression system, and purified using standard methods.

5. The luciferase and GFP polypeptides were shown to be located in the interior portions of the VLPs, and to retain their enzymatic activity. The VLPs were administered to cells by incubating purified VLPs with cultured HeLa cells. The VLPs were taken up by the cells, and the GFP polypeptides continued to retain their fluorimetric activity within the cells.

6. The results described herein were obtained between June 2003 and March 2005 in experiments conducted under my direct control, using procedures taught in the '530 Application. Related experiments conducted over the same time frame have yielded similar results for a variety of proteins using the same methods and the routine optimization of experimental conditions.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code. I further understand that, should any such willful, false statements be relied upon in assessing the patentability of the instant application, such statements may jeopardize the validity of the application or any patent issuing therefrom.

Respectfully submitted,

Dated: 5/11/08

By: _____
Leonard H. Rome, Ph.D.

EXHIBIT A

LEONARD H. ROME, PH.D.

Curriculum Vitae

Present Position:

Professor, Department of Biological Chemistry, David Geffen School of Medicine at UCLA
Senior Associate Dean for Research, David Geffen School of Medicine at UCLA
Interim Director, California NANOSystems Institute

Education:

B.S. 1971 (Chemistry)
The University of Michigan, Ann Arbor, Michigan
M.S. 1973 (Biological Chemistry)
The University of Michigan, Ann Arbor, Michigan
Ph.D. 1975 (Biological Chemistry)
The University of Michigan, Ann Arbor, Michigan

Professional Positions:

National Institutes of Health, Section on Human Biochemical Genetics, NIAMDD, Bethesda, Maryland.
Chemist, 1975, Staff Fellow, 1976-1979
UCLA School of Medicine, Department of Biological Chemistry
Assistant Professor, 1979-1984
Associate Professor, 1984-1988
Professor, 1988-present
Vice Chairman, 1989-1997
UCLA School of Medicine
Senior Associate Dean for Research, 1997 – present
Interim Director, California NANOSystems Institute, 2007 - present

Fellowships and Honors:

NIH Predoctoral Fellow, 1972-1975
NIH Staff Fellow, 1976-1979
March of Dimes, Basil O'Connor grant, 1981-1983
American Cancer Society Faculty Research Award, 1983-1988
UCLA School of Medicine Award for Excellence in Education, 1991
California State University, Northridge, Chem Club's 1998
Distinguished Lecturer of the Year

Professional Organizations:

American Society for Biochemistry & Molecular Biology
American Association for the Advancement of Science
American Society for Cell Biology
American Society for Neurochemistry (1980-1994)
American Association for Cancer Research (1985 – 2000)
Association of American Medical Colleges (AAMC)
Group on Research Advancement and Development (GRAND)
GRAND Steering Committee (1998-present)
GRAND Chair (2005-2006)

University Affiliations:

Jonsson Comprehensive Cancer Center (Director for Strategic Planning and Partnerships, 2005-present)
Jonsson Cancer Center Foundation (Secretary, 1997-present)
California NanoScience Institute (CNSI)
(Associate Director 2005 – present)
Institute for Pure and Applied Mathematics (IPAM)
(Member Board of Trustees, 2004- present)
Molecular Biology Institute
Brain Research Institute

Consulting:

Journal of Biological Chemistry, Editorial Board, 1988-1992
Developmental Neuroscience, Editorial Board, 1994-2002
Centaur Pharmaceuticals, 1998-2000
Eli Lilly Research Laboratories, 1997- 2001
California Science Center, Jury to select California Scientist of the Year (2000-present)

School of Medicine Service / Committee Memberships:

Vice Chairman, Department of Biological Chemistry (1989-1997)
Chair, Faculty Executive Committee, FEC (1995 - 1997); Served as Past Chair (1997-1998)
Member and Vice Chair Faculty Executive Committee, FEC (1992- 1995)
School of Medicine Educational Policy and Curriculum Committee (1986-1992)
Included: Steering Committee, Electives Subcommittee, Evaluation Subcommittee, Curriculum Review Subcommittee, Planning Committee for Honors and Thesis Program, Interactive Teaching Subcommittee (Committee Chair, 1987-1993)
Computer Network Committee (1988-1998)
Mental Retardation Research Center:
Research Advisory Committee (1988-1998)
Co-Director Media and Microcomputer Core (1989-1998)
Associate Director for Education (1994-1997)
Brain Research Institute:
Executive Advisory Committee (1988-1996)
H.W. Magoun Lectureship Selection Committee (1988-1996)
Associate Director for Education (1994-1995)
Academic Information Technology Committee (Co-Chair 1998 - present)
School of Medicine Nutrition Task Force (1989-1998)
Jonsson Comprehensive Cancer Center:
Basic Research Seed Grants Review Committee (1988-1997)
Cell Biology Program Area (Associate Director, 1991- 1995)
Molecular Cell & Developmental Biology Program Area (Director 1996-2003)
Cancer Cell Biology Program Area (Director 2003 - 2005)
Directors Advisory Review Committee (1997- present)

School of Medicine Service / Committee Memberships (continued):

Search Committees:

- Dean of the School of Medicine (Member, 1992 - 1994)
- Department of Psychiatry Chair and Director NPI (Chair,1989-1990)
- Director, Brain Research Institute (Member,1991)
- Chair, Department of Pathology (Member,1995)
- Two Assistant Professors, MRRC (Chair, 1995-1996)
- Director, Brain Research Institute and Head of the Neuroscience IDP (Member, 1995)
- Vice Chair, Department of Orthopedics (2006 – present)

Faculty Director HHMI Imaging facility (BTRIP) 1996-2003
Oversight responsibilities for Advanced Research Computing facility (ARC) 1998-2003
Oversight responsibilities for Computing Technologies Research Lab (CTRL) 2003-present
School of Medicine Safety Committee (Chair, 1997-present)
Planning Committee for the New Hospital

- Information Systems Planning Committee (1996)
- Research Planning Committee (1996)
- Steering Committee for the New Hospital- Master Planning Phase II (1996 - 2001)
- Master Planning Phase II, Research Committee (Co-Chair,1996 - 1998)
- East of Westwood Planning Committee (Chair, Research subcommittee 1998-2005)

Editorial Board UCLA Medicine Magazine (1998 – present)
Advisory Committee for Multimedia Education (1996 - 1998)
School of Medicine LCME Accreditation Task Force (1996-1997)
Capital Programs Committee (1997-present)
School of Medicine Space Committee (1998-present)
School of Medicine Planning Committee for NRB, RB2/Luck (1998-2006)
Member, Board of Directors, Gerald Oppenheimer Family Foundation for the Prevention of Eye Disease (2003-present)
School of Medicine Writing Requirement Task Force (2006 – present)
CTSA Section Chair (2005 – 2006)

Campus Wide Service / Committee Memberships:

UCLA Legislative Assembly (1982-1987)
Academic Planning and Budget Advisory and Coordinating Council (1995-1996)
Neuroscience IDP Advisory Committee (1992-1997)
Graduate Division Fellowship Review Committee (1993-present)
Instructional and Research Computing Committee (IRCC) (1994-1997)
Council of FEC Chairs (1994-1997)
UCLA Health Sciences Government Relations Task Force (1996-1997)
Chancellors Blue Ribbon Committee on Human Subject Protection (1996 - 1997)
Academic Information Technology Board (1997-1999)
Sepulveda VA Vervet Colony, Committee to establish a "Memorandum of Understanding" (1998-1999)
Research Service Advisory Board (1998 – 2000)
Graduate Division, Postdoctoral Advisory Committee (1998-present)

Campus Wide Service / Committee Memberships (continued...):

Task Force to evaluate combining of Vice Chancellor Research and Dean of the Graduate Division (1999)
Search Committees:
Vice Chancellor, Research (member, 1999 – 2000)
Dean, Physical Sciences (Chair, 2000 – 2001)
Dean, School of Engineering and Applied Science (member, 2001 – 2003)
Director, California NanoSystems Institute (Chair, 2002 – 2003)
Presentation on behalf of campus for the Life Sciences Initiative (LSI) Program, (Oakland CA, 1999)
EVC Hume Special Task Force on Technology Transfer (1999 - 2001)
UCLA Campus-wide IT Retreat (Presentation - Dec.1999)
EVC Hume Computer Science Task Force (2000 - 2001)
UCLA, Associate Vice Chancellor for Research for the Life and Health Sciences, 2001 -2004
California NANOSystems Institute – Budget Committee, (2003 – 2006)
California NANOSystems Institute – Membership Committee (2003 – 2006)
California NANOSystems Institute – Executive Committee (2003 – present)
California NANOSystems Institute – Governing Board (2004 – present)
California NANOSystems Institute – Interim Director (2007 – present)
Chancellors Competitiveness Task Force (2002- 2003)
Competitiveness Task Force Space Action Group (2002- 2003)
Competitiveness Task Force, Endowed Chairs and Graduate Student Fellowships Subcommittee (2003- 2004)
Human Subject Protection Grant (NIH) Steering Committee (Chair, 2002 – 2006)
Bioscience Initiative Advisory Committee (2003 – 2005)
Research Investment Committee (2003 – 2004)
Committee for the Donald J. Cram Chair in Organic Chemistry (2005 – present)
Vice Chancellor for Medical Sciences Strategic Planning Group (2005 – present)
Council of Biosciences Resources (Member 2006 – present)

School of Medicine Service - Teaching and Other:

Overall Departmental Course Chair for Medical Courses (1989-1997)
Course Co-Chair (with Dr. John Tormey) Clinical Applications of Basic Science (CAMBS) (1993-1995)
Course Chair Biological Chemistry for Medical Students (BC201) (1989-1990)
Course Chair Biological Chemistry 205C (Lab for Dental Students) (1989 - 1992)
Course Co-Chair (with Drs. J. Edmond and A. Van der Bliek, E. Ujita Lee) Human Biochemistry and Nutrition Laboratory (M204) (1985-2003)
M298 (Access Seminar Course) Fall 1998
Organizer 1992 Lucille P. Markey Trust Symposium "The RNA World"
Organizer 1998 Amgen/UCLA Symposium "Cutting Edge Research Leads to Novel Therapeutics"
Organizer 1998 Amersham Pharmacia Biotech / UCLA - "Technology Day"

School of Medicine Service - Teaching and Other (continued...):

Organizer "Biobasics" 1999 – present
Co-organizer (with Robert Goldberg) Keck Symposium on Bioengineered Foods (2003)

Outside Service:

Search Committee for ACOS Research at the GLA VA (1999 – 2000)
Congressional Liaison Committee and Joint Steering Committee for Public Policy (ASBMB, ASCB) (1997-present)
Moderator, 18th Annual AMA Science Reporters Conference (1999)
Speaker 2001 UCLA Foundation Retreat
Association of American Medical Colleges (AAMC)
- GRAND Steering Committee (1999-present)
- GRAND Steering Committee Chair (2005 – 2006)
- Presentation at 1999 Research Deans Meeting
- Session Moderator at 2001 Research Deans Meeting
- 2006 Distinguished Research Award Committee

Speaker 2002 National Youth Leadership Forum
Organizing Committee - International Vault Research Group, First International Meeting – May 16-18, 2002. Amsterdam, The Netherlands

Graduate Students Supervised:

Corinne Campbell	1980-1984; Ph.D. 1984.	Assistant Prof., Univ. Colorado, Denver, CO.
Karen Bame	1982-1985; Ph.D. 1985.	Professor, Univ. of Missouri, Kansas City, MO.
Michael Cardwell	1983-1988; Ph.D. 1988.	M.D. 1992. OB/GYN, Wisconsin.
Robert P. Searles	1982-1988; Ph.D. 1988.	Research Fellow, Oregon Regional Primate Center, Beaverton, OR
Steven Hamilton	1989-1993; Ph.D. 1994.	Assistant.Professor, Psychiatry, UCSF
Sanjay Vasu	1989-1994; Ph.D. 1994.	Scientist, Quorex Pharmaceuticals,Carlsbad, CA
Elisabeth Inman	1992-1997; Ph.D. 1997.	Senior Scientist, Focus Diagnostics, Inc.
Lucia Notterpek	1990-1994; Ph.D. 1994.	Associate Professor, University of Florida, College of Medicine
Steve Quinlivan	1995-1997; M.S. 1997.	Zymed Inc. Santa Clara, CA
Amara Siva	1995-2000; Ph.D. 2000.	Staff Scientist, Alexion Antibody Technologies
Nil Emre	1998-2004; Ph.D. 2004.	Post Doctoral Fellow, Scripps
Michael Poderycki	1999 – 2004; Ph.D. 2004	Post Doctoral Fellow, Scripps
Sun Mi Choi	2005 – 2006; M.S. 2006	Medical Student, LSU
Yvette Garcia	2002 – 2006; M.S. 2006	
Melody Pupols	2005 – present	
Daniel Buehler	2006 – present	

Postdoctoral Fellows Supervised:

Anton Steiner M.D. Ph.D.	1980-1981	Internist, Santa Monica, CA.

Postdoctoral Fellows Supervised (continued…):

Margarete Mehrabian Ph.D.	1981-1982	Research Faculty, UCLA.
Keith Westcott Ph.D.	1983-1985	Scientist, Amgen, Thousand Oaks, CA.
Nancy Kedersha Ph.D.	1984-86 and 88-90	Scientist, Harvard, Cambridge, MA.
Diane Chugani Ph.D.	1988-1989	Asst. Professor, Pediatrics, Wayne State, MI.
Ted Thederhan Ph.D.	1989-1990	Owner, Bremshield (a biotech company).
John Kasckow, M.D. Ph.D.	1990-1991	Psychiatry, UC Irvine.
Shahnaz Malek-Hedayat, Ph.D.	1988-1993	Pharmacist, Wayne State, MI..
Leena S. Sawant, Ph.D.	1995-1996	Leave of absence to start a family.
Valerie (Slagel) Kickhoefer, Ph.D.	1989-1994	Associate Research Biochemist, UCLA.
Beth Marbois, Ph.D.	1997-1999	Assistant Research Biochemist, UCLA.
Andrew Stephen, Ph.D.	1997-2000	Scientist, NCI-Frederick Cancer Research and Development Center
Sujna Raval, Ph.D.	1996-2002	Assistant Research Biochemist, UCLA.
Julie Akana, Ph.D.	2006-2007	
Upendra Kar, Ph.D.	2007-present	
Muri Han, Ph.D.	2007-present	
Jian Yang, Ph.D.	2008-present	

BIBLIOGRAPHY

Research Papers (Abstracts not included):

1. Rome, L.H. and Lands, W.E.M.: Properties of a partially-purified preparation of the prostaglandin-forming oxygenase from sheep vesicular gland. **Prostaglandins 10:** 813-824 (1975).
2. Rome, L.H. and Lands, W.E.M.: Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. **Proc. Natl. Acad. Sci. USA 72:** 4863-4865 (1975).
3. Rome, L.H., Lands, W.E.M., Roth, G.J. and Majerus, P.W.: Aspirin as a quantitative acetylating reagent for the fatty acid oxygenase that forms prostaglandins. **Prostaglandins 11:** 23-30 (1976).
4. Neufeld, E.F., Sando, G.N., Garvin, A.J. and Rome, L.H.: The transport of lysosomal enzymes. **J. Supramolec. Struct. 6:** 95-101 (1977).
5. Rome, L.H., Garvin, A.J. and Neufeld, E.F.: Human kidney α-L-iduronidase; Purification and characterization. **Arch. Biochem. Biophys. 189:** 344-353 (1978).
6. Rome, L.H., Garvin, A.J., Alietta, M. and Neufeld, E.F.: Two species of lysosomal organelles in cultured human fibroblasts. **Cell 17:** 143-153 (1979).
7. Rome, L.H., Wiessmann, B. and Neufeld, E.F.: Direct demonstration of binding of a lysosomal enzyme, α-L-iduronidase, to receptors on cultured fibroblasts. **Proc. Natl. Acad. Sci. USA 76:** 2331-2334 (1979).
8. Rome, L.H. and Miller, J.: Butanedione treatment reduces receptor binding of a lysosomal enzyme to cells and membranes. **Biochem. Biophys. Res. Commun 92**: 986-993 (1980).
9. Rome, L.H. and Crain, L.R.: Degradation of mucopolysaccharide in intact isolated lysosomes. **J. Biol. Chem. 256**: 681-687 (1981).
10. Steiner, A.W. and Rome, L.H.: Assay and purification of a solubilized membrane receptor that binds the lysosomal enzyme α-L-iduronidase. **Arch. Biochem. Biophys. 214**: 681-687 (1982).
11. Campbell, C.H., Fine, R.E., Squicciarini, J., and Rome, L.H.: Coated vesicles from rat liver and calf brain contain cryptic mannose 6-phosphate receptors. **J. Biol. Chem. 258**: 2628-2633 (1983).
12. Rome, L.H., Hill, D.F., Bame, K.J. and Crain, L.R.: Utilization of exogenously added acetyl coenzyme A by intact isolated lysosomes. **J. Biol. Chem. 258**: 3006-3011 (1983).
13. Campbell, C.H., Miller, A.L., and Rome, L.H.: Incorporation of mannose 6-phosphate receptors into liposomes: Receptor topography and binding of α-mannosidase. **Biochem. J. 214**: 413-419 (1983).
14. Campbell, C.H. and Rome, L.H.: Coated vesicles from rat liver and calf brain contain lysosomal enzymes bound to mannose 6-phosphate receptors. **J. Biol. Chem. 258**: 13347-13352 (1983).
15. Berg, R.A., Schwartz, M.L., Rome, L.H. and Crystal, R.G.: Lysosomal function in the degradation of defective collagen in cultured lung fibroblasts. **Biochemistry 23**: 2134-2138 (1984).

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16. Mehrabian, M., Bame, K.J. and Rome, L.H.: Interaction of rat liver lysosomal membranes with actin. **J. Cell Biol. 99**: 680-685 (1984).
17. Hill, D.F., Bullock, P.N., Chaippelli, F. and Rome, L.H.: Binding and internalization of lysosomal enzymes by primary cultures of rat glia. **J. Neurosci. Res. 14**: 35-47 (1985).
18. Bame, K.J. and Rome, L.H.: Acetyl-CoA: α-glucosaminide N-acetyltransferase. Evidence for a transmembrane acetylation mechanism. **J. Biol. Chem. 260**: 11293-11299 (1985).
19. Rome, L.H., Bullock, P.N., Chaippelli, F., Cardwell, M.C., Adinolfi, A.M. and Swanson-Hayes, D.: Synthesis of a myelin-like membrane by oligodendrocytes in culture. **J. Neurosci. Res. 15**: 49-65 (1986).
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21. Kedersha, N.L., Hill, D.F., Kronquist, K.E. and Rome, L.H.: Subpopulations of liver coated vesicles resolved by preparative agarose gel electrophoresis. **J. Cell Biol. 103**: 287-297 (1986).
22. Kedersha, N.L. and Rome, L.H.: Preparative agarose gel electrophoresis for the purification of small organelles and particles. **Anal. Biochem. 156**: 161-170 (1986).
23. Tietze, F., Rome, L.H., Butler, J.DeB., Haper, G.S. and Gahl, W.A.: Impaired clearance of free cysteine from lysosome-enriched granular fractions of I-cell disease fibroblasts. **Biochem. J. 237**: 9-15 (1986).
24. Bame, K.J. and Rome, L.H.: Acetyl-CoA: α-glucosaminide N-acetyltransferase. Evidence for an active site histidine residue. **J. Biol. Chem. 261**:10127-10132 (1986).
25. Kedersha, N.L. and Rome, L.H.: Isolation and characterization of a novel ribonucleoprotein particle: Large structures contain a single species of small RNA. **J. Cell Biol. 103**: 699-709 (1986).
26. Bame, K.J., and Rome, L.H.: Genetic evidence for transmembrane acetylation by lysosomes. **Science 233**: 1087-1089 (1986).
27. Westcott, K.R., Searles, R.P. and Rome, L.H.: Detection of ligand- and pH-dependent conformation changes in liposome-associated mannose 6-phosphate receptor. **J. Biol. Chem. 262**: 6101-6107 (1987).
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29. Westcott, K.R. and Rome, L.H.: Cation-independent mannose 6-phosphate receptor contains covalently bound fatty acid. **J. Cell. Biochem. 38**: 23-33 (1988).
30. Cardwell, M.C., and Rome, L.H.: Evidence that an RGD-dependent receptor mediates the binding of oligodendrocytes to a novel ligand in a glial-derived matrix. **J. Cell Biol. 107**: 1541-1549 (1988).
31. Cardwell, M. C. and Rome, L.H.: RGD-containing peptides inhibit the synthesis of myelin-like membranes by cultured oligodendrocytes. **J. Cell Biol. 107**: 1551-1559 (1988).

Research Papers (continued):

32. Kedersha, N.L., Miquel, M-C., Bittner, D. and Rome, L.H.: Vaults. II. Ribonucleoprotein structures are highly conserved among higher and lower eukaryotes. **J. Cell Biol. 110**: 895-901 (1990).
33. Kedersha, N.L. and Rome L.H.: Vaults: Large cytoplasmic RNP's that associate with cytoskeletal elements. **Molec. Biol. Reports 14**: 121-122 (1990).
34. Bullock, P.N. and Rome, L.H.: Glass micro-fibers: A model system for study of early events in myelination. **J. Neurosci. Res. 27:** 383-393 (1990).
35. Chugani, D.C., Kedersha, N.L. and Rome, L.H.: Vault immunofluorescence in brain: New insights regarding the origin of microglia. **J. Neuroscience 11**: 256-268 (1991).
36. Kedersha, N.L., Heuser, J.E., Chugani, D.C. and Rome, L.H.: Vaults. III. Vault Ribonucleoprotein Particles Open into Flower-like Structures with Octagonal Symmetry **J. Cell Biol. 112:** 225-235 (1991).
37. Malek-Hedayat, S. and Rome, L.H.: Expression of Multiple Integrins and Extracellular Matrix Components by C6 Glioma Cells **J. Neurochem. Res. 31:** 470-478 (1992).
38. Kickhoefer, V.A. Searles, R.P., Kedersha, N.L., Garber, M.E., Johnson, D.L. and Rome, L.H.: Vault RNP particles from rat and bullfrog contain a related small RNA that is transcribed by RNA polymerase III. **J. Biol. Chem. 268:** 7868-78173 (1993).
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42. Notterpek, L., Bullock, P.N., Malek-Hedayat, S., Fisher, R., and Rome, L.H.: Myelination in Cerebellar Slice Cultures: Development of a System Amenable to Biochemical Analysis. **J. Neurosci. Res. 36:**621-634 (1993).
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45. Notterpek, L. and Rome L.H.: Functional Evidence for the Role of Axolemma in CNS Myelination. **Neuron 13:**473-485 (1994).
46. Vilalta, A., Kickhoefer, V.A., Rome, L.H., and Johnson, D.L.: The rat vault RNA gene contains a unique RNA polymerase III promoter composed of both external and internal elements that function synergistically. **J. Biol. Chem 269:**29752-29769 (1994).
47. Kickhoefer, V.A. and Rome, L.H.: The sequence of a cDNA encoding the major vault protein from *Rattus norvegicus*. **Gene 151:**257-260 (1994).

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48. Notterpek, L. and Rome L.H.: A Protein Involved in CNS Myelination: Localization to the ECM and Induction in Neuroblastoma Cells. **Developmental Neuroscience 16:** 267-278 (1994).
49. Malek-Hedayat, S. and Rome, L.H.: Molecular cloning and sequence of the cDNA encoding the rat oligodendrocyte integrin β_1 subunit. **Gene 158:** 287-290 (1995).
50. Vasu, S. and Rome, L.H.:*Dictyostelium* vaults: Disruption of the major proteins reveals growth and morphological defects and uncovers a new associated protein. **J. Biol. Chem. 270:** 16588-16594 (1995).
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52. Kickhoefer, V.A., Rajavel, K.S., Scheffer, G.L., Dalton, W.S., Scheper, R.J., and Rome, L.H.: Vaults are up-regulated in multidrug resistant cancer cell lines. **J. Biol. Chem. 273**: 8971-8974 (1998).
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55. Herrmann, C., Golkaramnay, E., Inman, E., Rome, L., and Volknandt, W.: Recombinant Major Vault Protein is Targeted to Neuritic Tips of PC12 Cells. **J. Cell Biol. 144**:1163-1172 (1999).
56. Raval-Fernandes, S.D., Kickhoefer, V.A. and Rome, L.H.: Cloning of a cDNA encoding a sequence-specific single-stranded-DNA-binding protein from *Rattus norvegicus.*: **Gene 237:**201-207 (1999).
57. Kickhoefer, V.A., Siva, A.C., Kedersha, N.L., Inman, E.M., Ruland, C., Streuli, M., Rome, L.H.: The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase. **J. Cell Biol. 146**:917-28 (1999).
58. Kickhoefer, V.A., Stephen, A.G., Harrington, L., Robinson, M.O. and Rome, L.H.: Vaults and Telomerase Share a Common Subunit, TEP1. **J. Biol. Chem. 274**: 32712-32718 (1999).
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EXHIBIT B

Engineering of vault nanocapsules with enzymatic and fluorescent properties

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One of the central issues facing the emerging field of nanotechnology is cellular compatibility. Nanoparticles have been proposed for diagnostic and therapeutic applications, including drug delivery, gene therapy, biological sensors, and controlled catalysis. Viruses, liposomes, peptides, and synthetic and natural polymers have been engineered for these applications, yet significant limitations continue to prevent their use. Avoidance of the body's natural immune system, lack of targeting specificity, and the inability to control packaging and release are remaining obstacles. We have explored the use of a naturally occurring cellular nanoparticle known as the vault, which is named for its morphology with multiple arches reminiscent of cathedral ceilings. Vaults are 13-MDa ribonucleoprotein particles with an internal cavity large enough to sequester hundreds of proteins. Here, we report a strategy to target and sequester biologically active materials within the vault cavity. Attachment of a vault-targeting peptide to two proteins, luciferase and a variant of GFP, resulted in their sequestration within the vault cavity. The targeted proteins confer enzymatic and fluorescent properties on the recombinant vaults, both of which can be detected by their emission of light. The modified vaults are compatible with living cells. The ability to engineer vault particles with designed properties and functionalities represents an important step toward development of a biocompatible nanocapsule.

capsule | nanoparticle

Vaults are large ribonucleoprotein particles that exist as naturally occurring nanocapsules with a thin (≈20 Å) protein shell surrounding an internal cavity of $5 \times 10^7 Å^3$ (1) (Fig. 1). The molecular mass of the vault is 12.9 ± 1 MDa. Cryoelectron microscopy (cryoEM) single-particle reconstruction has revealed the vault to be a hollow, barrel-like structure with two protruding caps and an invaginated waist with overall dimensions of 420 × 420 × 750 Å (2), larger in mass and size than some icosahedral viruses. Although many different roles have been proposed for the vault since its first description in 1986 (3), including nucleocytoplasmic transport and sequestration of molecular cargo, its cellular function remains unknown. The vault is the largest known cellular ribonucleoprotein particle, yet it has a relatively simple molecular composition with multiple copies of just three proteins, the 100-kDa major vault protein (MVP), the 193-kDa vault poly(ADP ribose) polymerase (VPARP), and the 290-kDa telomerase-associated protein 1, and one to three distinct untranslated RNA molecules (4). The MVP is presumed to be present in 96 copies per vault, based on the observed symmetry of the particle and the estimate that MVP accounts for ≈75% of the total protein mass in the particle. Expression of MVP in insect cells, by using the baculovirus expression system, revealed that this protein alone is capable of directing the formation of recombinant vault particles with a structure similar to endogenous particles (5).

A wide variety of strategies for encapsulation of biomaterials exist, including synthetic and natural polymers, hydrogels, peptides, liposomes, and viruses. However, the use of these materials in biological systems is often limited by poor biocompatibility, immune responses, lack of targeting specificity, and the inability to control packaging and release. We have been analyzing the use of recombinant vaults to overcome some or all of these limitations. Here, we demonstrate that, by using a vault-targeting peptide, a heterologous protein can be functionally sequestered within the central cavity of vault particles, and that these modified vaults are compatible with living cells.

Methods

Vault-Targeting Constructs and Purification. The luciferase-encoding DNA (6) was PCR-amplified from pGL3basic (Promega), fused to the vault interaction domain derived from VPARP (INT) (GenBank accession no. AF158255, amino acids 1471–1724) and inserted into the baculovirus expression vector, pFASTBAC (Invitrogen). The INT domain is defined as the 162-aa region at the C terminus of VPARP (amino acids 1563–1724), which is the smallest region identified for interaction with MVP. The expressed fusion domain is slightly larger (254-aa) but includes the full INT domain. The gene encoding a variant GFP, green lantern (GL) (Invitrogen) (7), was PCR-amplified, fused to either an INT domain (amino acids 1471–1724) or the N terminus of the cDNA-encoding rat MVP (GenBank accession no. U09870), and inserted into pFASTBAC. Using the Bac-to-Bac expression system (Invitrogen), recombinant insect viruses were generated. Sf9 cells were infected with the recombinant INT baculoviruses alone or in combination with recombinant tagged MVP baculoviruses, and recombinant vaults were purified as described in ref. 5. Immunoblot analyses were carried out by using either anti-MVP (8) or anti-VPARP (9) polyclonal antibodies. Note that the anti-VPARP antibody recognizes the INT domain of VPARP.

CryoEM Single-Particle Reconstruction. The luc-INT vault reconstruction at 28-Å resolution was based on 661 particle images collected on a CM120 cryotransmission electron microscope (FEI, Hillsboro, OR). CryoEM and single-particle reconstruction were performed as described in refs. 1, 2, and 10. The resolution was determined with the Fourier shell correlation 0.5 threshold criterion (11). The difference density was calculated by using the AVS software package (Advanced Visual Systems, Waltham, MA) after filtering the two input reconstructions to the same resolution.

Luciferase Enzyme Assays. Purified luciferase (Promega) was diluted to 47 nM with 1× PBS (Fisher Scientific) before assaying. Luciferase assay substrate was prepared as described in ref. 12.

Abbreviations: cryoEM, cryoelectron microscopy; MVP, major vault protein; VPARP, vault poly(ADP-ribose) polymerase; INT, vault interaction domain derived from VPARP; luc-INT, luciferase/INT fusion protein; GL, green lantern.

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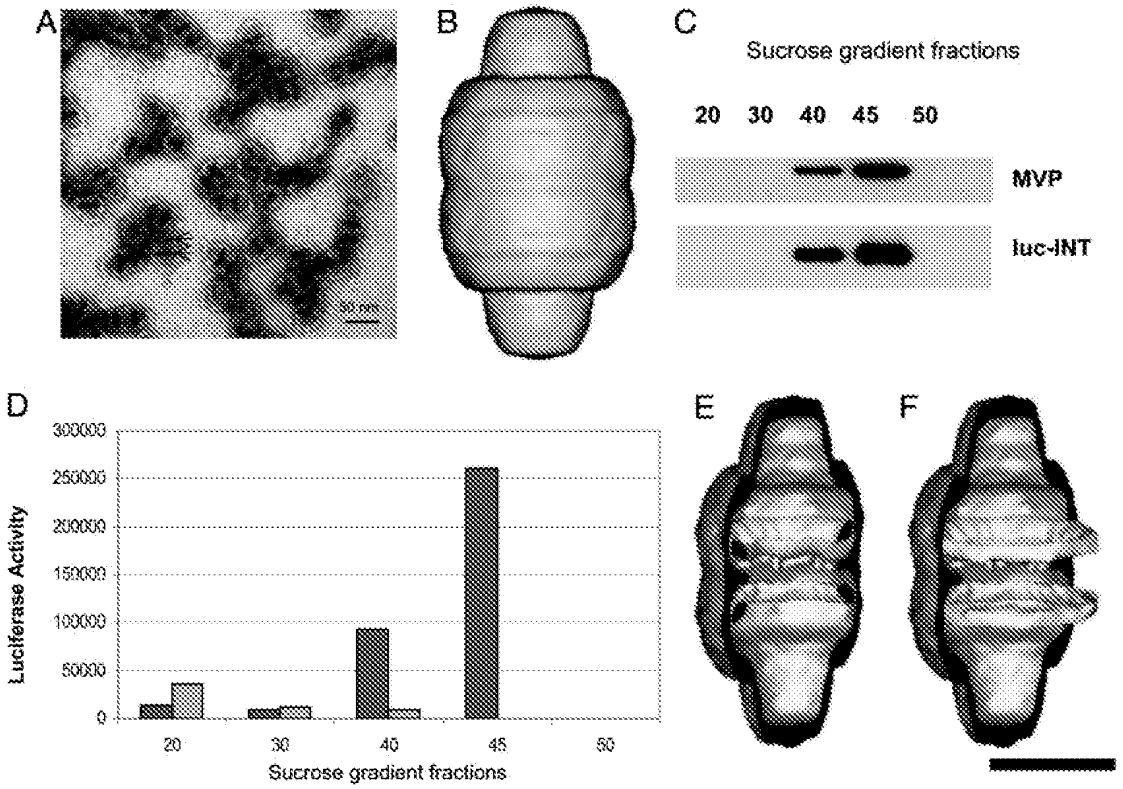

Fig. 1. Purification, EM, and cryoEM single-particle reconstruction of the luc-INT vaults and localization of the luc-INT density. (*A*) An electron micrograph of negative-stained vault particles containing MVP and luc-INT. The arrows indicate the darkly staining bands across the central barrel. (Scale bar, 50 nm.) (*B*) The luc-INT vault reconstruction at a resolution of 28 Å based on 661 particle images collected on a CM120 cryotransmission electron microscope (FEI). (*C* and *D*) Sucrose gradient fractions were immunoblotted to detect the indicated protein species in the final step of luc-INT vault purification indicating their copurification. Likewise, luciferase activity peaks with the purified particles in the 40% and 45% fractions. Blue bars show the luc-INT vaults, and gold bars show luc-INT alone. (*E* and *F*) Difference density assigned to luc-INT (gold) superimposed on a reconstruction of a vault formed by MVP alone (blue). Both density maps are shown cropped lengthwise. The MVP vault reconstruction is that of HisT7-MVP (10). (*F*) The full luc-INT density rings (gold) are shown superimposed on the cropped MVP vault (blue). (Scale bar, 25 nm.)

Briefly, the substrate contained 25 mM Gly-Gly, 15 mM K_2PO_4, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT, 0.1 mM CoA, and 75 μM luciferin (pH 8.0). In a glass vial, 50 μl of either standard luciferase or sample solution was added, and 100 μl of the luciferase substrate was added in the dark a few seconds after data collection had begun. The glass vial was placed at the entrance slit of a single monochromator, which dispersed the captured luminescence onto an intensified charge-coupled device camera (PI-MAX, Princeton Instruments, Trenton, NJ). The camera was programmed to collect a spectrum every 500 ms for 5 min. The software was programmed to integrate the area under the luminescence peak from 540 to 575 nm for each spectrum that was collected. These areas were then plotted vs. time with EXCEL (Microsoft) or IGOR PRO software (WaveMetrics, Lake Oswego, OR).

For the preincubation experiments, 10 μl of 20 mM ATP or 10 μl of 750 μM luciferin was added to 50 μl of luc-int/MVP vaults. The mixture was incubated on ice for $\approx$40 min before being transferred to a glass vial. Luciferase substrate (90 μl) containing either no ATP or luciferin was added to the glass vial for data collection as described above.

Green Fluorescence Quenching. GL-INT vaults (0.01 mg/ml) in Mes buffer (20 mM, pH 6.5) were added to a glass vial, an equal volume of quencher solution (1 M KCl or KNO_3 in 20 mM Mes buffer, pH 6.5) was added, and the intensity was monitored as a function of time. For the Stern–Volmer data, variable concentrations of Congo red in 20 mM Mes buffer (pH 6.5) were used, and the fluorescence intensity was monitored. The fluorescence was recorded by using a monochromator (HR-320, Instruments SA, Edison, NJ) and a gated intensified charge-coupled device camera (PI-MAX). The intensity was monitored by using the gated intensified charge-coupled device camera, where the integration time was 0.5 s and each integration followed directly after the previous integration. The same experiment was performed by using free GL-INT and GL-INT vaults. Samples were excited by using an argon ion laser (Innova 90C-5, Coherent Radiation, Palo Alto, CA). The excitation wavelength and power were 457 nm and 10 mW, respectively.

Uptake of GL-INT Vaults by HeLa Cells. HeLa cells were grown on four-well chamber slides (Lab Tek II, Nalge). Purified GL-INT vaults (10 μg) were added to the HeLa cell monolayer and incubated for 1 h at 37°C. The cells were washed with PBS, incubated with cholera toxin B/Alexa Fluor 594 conjugate (Molecular Probes) for 10 min at 12°C to stain cell membranes, washed a second time, and covered with glass coverslips, and live cells were visualized by using confocal microscopy (green HeNe, 594 nm; argon, 488 nm; 0.2-μm slices).

Results

Targeting Heterologous Proteins into the Central Cavity of Recombinant Vaults. INT was previously identified as being responsible for its interaction with MVP (9). We reasoned that, because this domain is responsible for VPARP assembly into vaults, INT could be used to target heterologous non-vault proteins into vault particles. We demonstrate the use of this targeting peptide with two proteins whose presence is revealed by light emission,

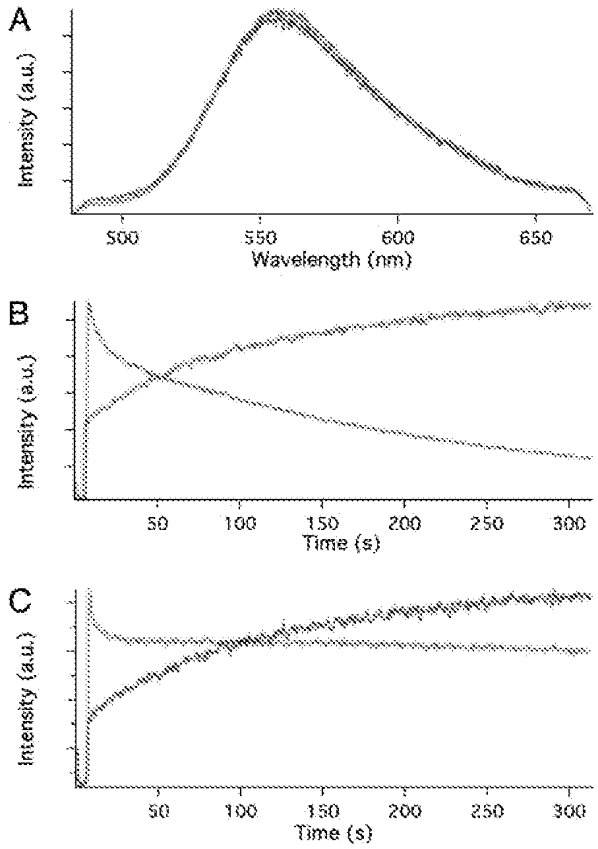

Fig. 2. Analysis of the luc-INT enzyme reaction. (*A*) Superimposed emission spectra produced by the action of INT-derivatized luciferase in solution (blue trace) and engineered into the interior of vaults (red trace). (*B*) Time dependence of the reaction product emission intensity from a solution of INT-derivatized luciferase (blue trace) and from luciferase engineered into the interior of vaults (red trace). Note the slow decay of the intensity at long times caused by the activity of the soluble enzyme and the gradual rise at long times caused by the sequestered enzyme. (*C*) Time dependence of the emission intensity from suspensions of engineered vaults. Vaults that are preincubated in ATP solution (red trace) produce product in a time dependence similar to that from soluble enzyme (compare with blue trace in *B*), whereas vaults preincubated in luciferin solution (blue trace) produce product in a time dependence similar to that from vaults that were not pretreated (compare with red trace in *B*).

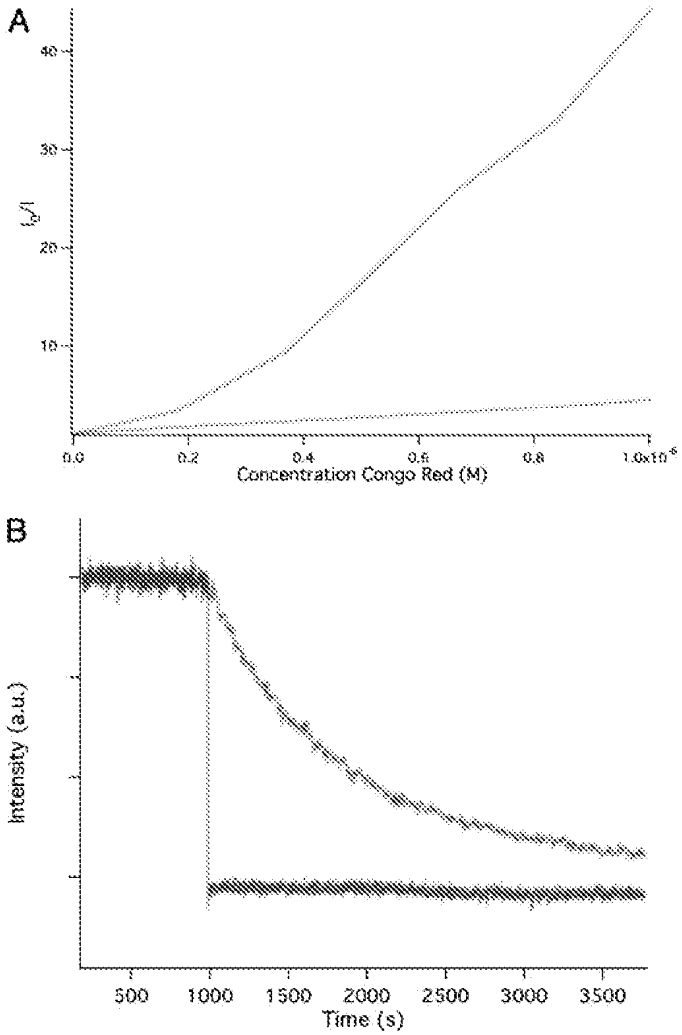

Fig. 3. Analysis of the GL-INT fluorescent properties. (*A*) Stern–Volmer plot for GL-INT (blue) and GL-INT vaults (red). The fluorescence intensity without quencher (I_o) divided by the intensity with quencher (I) is plotted against the Congo red concentration. Quenching is more efficient (demonstrated by the steep slope) when the fluorescent protein is sequestered within vaults. (*B*) Quenching of GL-INT fluorescence by KCl. The fluorescence of dissolved GL-INT is quenched immediately after addition of the salt at $t = 1{,}000$ sec (blue trace), but that of GL sequestered in vaults exhibits a much longer quenching time (red trace).

the enzyme luciferase (6) and a variant of the fluorochrome-containing GFP (7). The INT domain was fused to the C terminus of luciferase. When the luciferase/INT fusion protein (luc-INT) was expressed in insect cells by using the baculovirus system, the modified protein retained enzymatic activity and remained in the soluble fraction. In contrast, coexpression of luc-INT and MVP in insect cells altered the luc-INT fractionation profile, resulting in its sedimentation at 100,000 × *g*, indicative of assembly into vaults. Analysis of the recombinant vaults (hereafter referred to as luc-INT vaults) after purification on a sucrose gradient demonstrated that both the luc-INT protein (Fig. 1*C*) and luciferase enzymatic activity cofractionate with MVP (Fig. 1*D*). An examination by negative-stain EM of the material purifying in the 40% and 45% sucrose fractions demonstrated abundant, regular vault-like particles (Fig. 1*A*). The recombinant luc-INT vault particles appear similar to endogenous vaults except for the presence of two prominent, darkly staining stripes across the central barrel (Fig. 1*A*, arrows).

The luc-INT protein was localized to the inner surface of the recombinant vault by using cryoEM single-particle reconstruction. Particle images of 661 luc-INT vaults were selected from cryomicrographs and processed to yield a 3D reconstruction at a resolution of 28 Å (Fig. 1*B*). The outer surface of this reconstruction has the characteristic features of other published vault reconstructions (1, 2, 10). Difference-mapping between reconstructions of the luc-INT vault and recombinant vaults formed from MVP alone revealed two internal bands of density attributed to multiple copies of the luc-INT protein (Fig. 1 *E* and *F*). The internal density bands are found ≈70 Å on either side of the central waist of the particle, adjacent to the location where the stripes were seen in the negative-stain images (Fig. 1*A*). The cryoEM reconstruction and difference mapping indicate that the luciferase enzyme is packaged into the cavity of the vault particle and targeted to the inner surface of the vault by the INT domain derived from the VPARP vault protein.

Functional Sequesteration of Heterologous Proteins *in Vitro* and *in Vivo*. We compared the time dependence of chemiluminescence intensity of the luciferase reaction products from soluble luc-

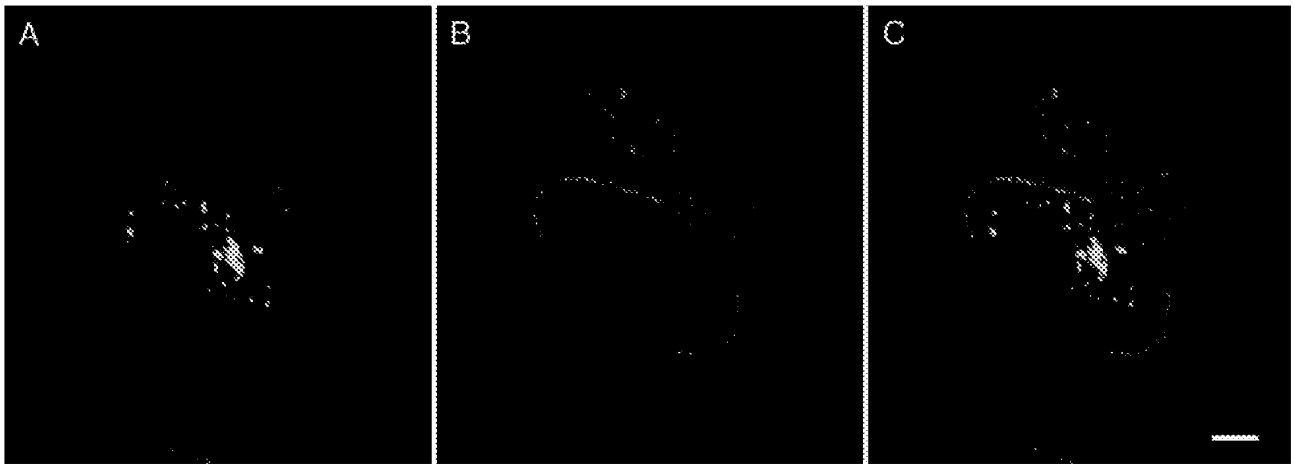

Fig. 4. GL-INT vault uptake in HeLa cells. (*A*) GL-INT vaults (green) observed on the membrane and inside the cell. (*B*) Membrane staining (red) observed with cholera toxin B/Alexa Fluor 594 conjugate. (*C*) A merge of *A* and *B* showing green punctate fluorescence inside the cells. (Scale bar, 8 μm.) The HeLa cells did not display any green fluorescence in the absence of added GL-INT vaults.

INT and luc-INT vaults by monitoring their emission at the 558-nm band maximum. The product spectra produced by the free, soluble luc-INT protein (Fig. 2*A*, blue trace) were virtually identical to those of the products produced by the enzyme sequestered in the luc-INT vaults (Fig. 2*A*, red trace). When the luciferase substrates luciferin and ATP were added to a solution containing soluble luc-INT, the emission intensity of the product increased to a maximum within a few seconds and was followed by a gradual decrease over hundreds of seconds (Fig. 2*B*, blue trace). In contrast, when the substrates were added to a suspension of luc-INT vaults containing sequestered luciferase, the intensity had an initial rapid rise but then rose gradually to a maximum in ≈300 sec (Fig. 2*B*, red trace). When the luc-INT vaults were preincubated in the presence of ATP and then exposed to luciferin (Fig. 2*C*, red trace), the time dependence was similar to that of the soluble luc-INT protein, displaying a fast rise in the emission intensity. Alternately, when luc-INT vaults were preincubated with luciferin and then exposed to ATP (Fig. 2*C*, blue trace), the emission intensity exhibited a slow increase over ≈300 sec, comparable with the time dependence of vaults exposed to both substrates simultaneously. Thus, the delayed maximum light emission catalyzed by the sequestered luciferase may be attributed to slow accumulation of ATP into the vaults. Perhaps either the charge or the larger size of ATP (507 vs. 248 Da for the neutral luciferin) is the reason behind the time delay in reaching the sequestered enzyme.

The generality of this approach was demonstrated by a second application with the GL protein, a variant of the GFP (7). GL-INT was expressed in insect cells by using the baculovirus system and was found to be highly fluorescent, with a band maximum at 510 nm. When the GL-INT protein is coexpressed with MVP in insect cells, recombinant vault particles containing both proteins, referred to as GL-INT vaults, can be purified. Evidence for this packaging is twofold. First, the sucrose gradient-purified vault pellet is bright green. Second, negative-stain EM demonstrated that these particles contain additional internal density similar to that of luc-INT vaults (data not shown).

Three spectroscopic studies verify that the GL-INT protein is packaged into vaults. First, fluorescence is observed only from the vaults themselves and not from the buffer; after multiple cycles of washing and ultracentrifugation followed by resuspension, fluorescence is observed from the resuspended vaults but not from the wash solvent. The fluorescence band maximum of the GL-INT vaults is 510 nm, identical to that of the soluble GL-INT protein. Second, Stern–Volmer kinetic analysis of quenching of the fluorescence of GL-INT vaults by Congo red revealed a very large quenching constant indicative of "superquenching" (13, 14) (Fig. 3*A*). This effect, a result of through-space Förster quenching, occurs when one quencher molecule acts on closely spaced donor molecules. This quenching effect suggests that in the GL-INT vaults, multiple copies of the GL-INT protein are brought into close proximity within the central barrel of the vault. Third, and most importantly, GL-INT associated with vaults is protected from the outside environment. Ionic strength affects the GL absorption spectrum (15). The addition of KNO_3 (16) or KCl to free GL-INT in solution causes immediate loss of fluorescence intensity (Fig. 3*B*, blue trace). In contrast, the addition of KCl to a suspension of GL-INT vaults causes a gradual decrease of the intensity (Fig. 3*B*, red trace). The quenching of GL-INT vaults by KCl requires >500 sec to reach the half-maximum value. The long quenching time indicates that GL-INT associated with vaults is protected

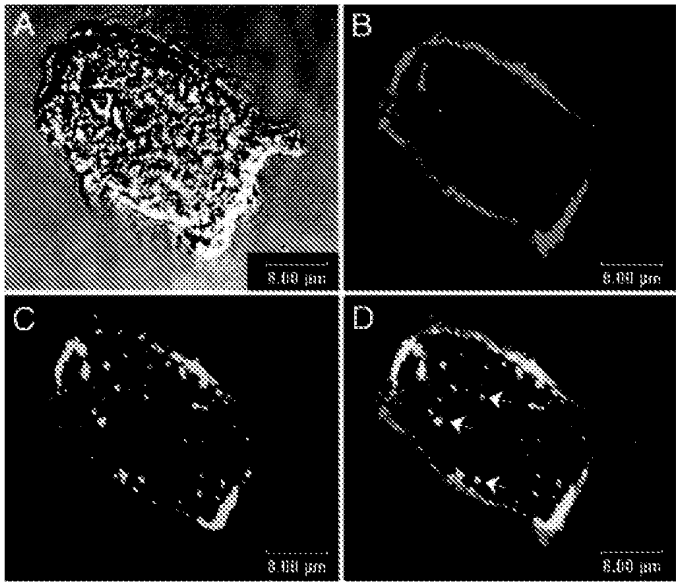

Fig. 5. GL-MVP vault uptake in HeLa cells. (*A*) DiC showing the HeLa cell. (*B*) Mostly membrane staining (red) seen with cholera toxin B/Alexa Fluor 594 conjugate. (*C*) Green fluorescent vaults seen on the membrane and inside the cells. (*D*) A merge of *B* and *C* showing vaults (green) inside the cells.

from the quenching ions. These spectroscopic observations suggest that GL-INT is packaged within the central cavity of recombinant vault particles, consistent with our cryoEM observations for luc-INT associated with vaults.

To determine whether recombinant vault nanocapsules are compatible with living cells and continue to sequester their contents within the cellular environment, we analyzed their uptake in cultured cells. Purified GL-INT vaults were added to cultured HeLa cells and incubated for 1 h, and their uptake was monitored by using confocal microscopy. A punctate pattern of green fluorescence was observed inside of the cells (Fig. 4). A similar punctate pattern was observed after uptake of GL-MVP vaults, formed from MVP tagged at the N terminus with GL (Fig. 5). In the case of GL-MVP vaults, the fluorescent protein is covalently linked to the vault. The comparable fluorescence patterns observed for both GL-INT and GL-MVP vaults suggest that the GL-INT vault particles remain intact after uptake into the cell.

Discussion

These studies demonstrate that recombinant vaults can be produced with chemically active or fluorescent proteins sequestered within the particle cavity. The baculovirus system is robust, allowing for production and purification of 4–20 mg of vaults per liter culture of cells. The INT domain appears to be a general targeting sequence that should be able to direct a wide variety of recombinant proteins (and other molecules) into the vault. Although we have not determined the precise stoichiometry of the luc-INT protein in the vault, the INT domain was initially identified in a yeast two-hybrid screen that used MVP as the bait (9). Therefore, the minimum INT binding domain is a MVP monomer. Thus, the maximum number of INT binding sites per vault would be 96 because there are 96 MVPs per particle. Because the internal volume of the vault is 5×10^7 Å^3, there is sufficient space for hundreds of proteins. Therefore, the maximum binding would depend on the size of the INT-tagged fusion protein, the spacing of the MVP binding sites, and any steric constraints that result from binding. The largest non-vault protein thus far targeted to the vault interior by using the INT domain is the ≈61-kDa firefly luciferase protein. Once targeted inside the vault, INT fusion proteins appear to be both functional and stably associated. This strategy could be used to confer unique properties onto vaults by targeting other molecules (e.g., metals, nucleic acids, polynucleotides, polymers, etc.) by virtue of fusing their protein binding domains onto the INT domain. Although the thin protein shell of the vault does not prevent the entry of small ligands, there appears to be a diffusion barrier, particularly for charged molecules. The entry of charged molecules into the vault cavity might require relatively slow conformational changes to take place within the vault protein shell. This behavior is consistent with recent *in vitro* assembly studies that demonstrate the vault protein shell to be a dynamic structure allowing the incorporation of the large vault associated proteins VPARP and telomerase-associated protein 1 into its interior (17).

The engineering of vault particles with designed properties and functionalities represents an important direction for the emerging field of bionanotechnology. This research establishes that intrinsic optical properties of proteins can be retained when they are confined within the vault nanocapsule. We expect that a wide range of proteins with other chemical properties may be sequestered within recombinant vaults by using this approach. We envision a number of applications for these engineered particles, including biologically based chemical sensors, bioreactors, and protein stabilizers, all of which can be targeted at the cellular level because of the biocompatibility of the capsule.

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